1355-Pos Board B247
The Most Recent, Catalytically Fit HDV Ribozyme Exhibits Minimal Global and Small-Scale Conformational Change upon Cleavage
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Historically, all small catalytic RNAs have been shown to undergo global conformational changes upon phosphodiester cleavage. However, the most recent of numerous hepatitis delta virus (HDV) ribozyme crystal structures has challenged this trend, as this crystal structure suggests that the precursor structure is already product-like in conformation. To further investigate this unusual observation, we have extensively characterized the solution behavior of several three-stranded versions of the HDV ribozyme from the recent crystal structure. Fluorescence gel shift assays show that varying lengths of the 5'overhang sequence adjacent to the active site result in the same degree of cleavage, whereas noncleavable substrates exhibit significantly more heterogeneity. Complementary steady state and time-resolved FRET assays demonstrated that the length of the 5'overhang sequence adjacent to the cleavage site affects the rates of conformational change upon substrate binding and cleavage. Molecular dynamics (MD) simulations were also performed to gain insight into the atomic behavior and catalytic relevance of the HDV ribozyme from the Chen et al crystal structure. These simulations suggested that the dU-1dG1dG2 motif used in the crystal does not result in a catalytically fit ribozyme compared to an all-ribose construct. Furthermore, altered active site conditions also result in lowered catalytic fitness. These simulations suggested that catalytic fitness is greatly disrupted by deprotonation of C75, supporting the hypothesis of the role of C75 as a general acid. Simulations also showed that the magnesium ion resolved near the scissile phosphate results in favorable catalytic geometry compared to simulations neutralized with sodium. Our experimental results demonstrate that, despite previously published results, all forms of the HDV ribozyme undergo significant global conformational changes upon self-cleavage, and our simulations show that C75 is poised to act as a general acid during cleavage.

1356-Pos Board B248
miR-122: An Antiviral Target against Hepatitis C Virus
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microRNA-122 (miR-122), a liver specific microRNA, has been shown to facilitate the Hepatitis C virus (HCV) replication and/or translation. Although the exact role played by miR-122 in this process is not fully understood, it has been shown that one of the functions of miR-122 is to stabilize the HCV RNA genome upon binding. There are two miR-122 binding sites within the HCV genome 5'-untranslated region (5'-UTR), named the S1 and S2 sites, both containing the miR-122 seed sequence. It has been shown that miR-122 is a valid antiviral target, as locked nucleic acids developed against miR-122 abolished the HCV replication. However, miR-122 has numerous other functions in the hepatic cell, which will also be affected by these LNAs. In this study, we adopted a different approach, namely to design peptide nucleic acids (PNAs) against the miR-122 binding sites within the HCV genome, sites conserved in all HCV genotypes and to test their antiviral properties. Our results indicate that one such PNA designed against the site S2 binds to the 5'-UTR of the HCV genome. And moreover, has the ability to invade the miR-122/HCV 5'UTR complex, releasing miR-122.

1357-Pos Board B249
PSD-95 MRNA Translation Regulation by the Fragile X Mental Retardation Protein
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Fragile X syndrome (FXS) is an inherited disease caused by a trinucleotide expansion on X the chromosome that silences the expression of the Fragile X Mental Retardation protein (FMRP). Individuals affected by FXS show different intensity of mental impairment and learning disabilities depending on the mutation severity. FMRP is an RNA binding protein which has been shown to bind to a G rich region of PSD-95 mRNA that encodes for Post Synaptic Density Protein (PSD-95). It has been shown that in complex with a specific microRNA (miR-125a) FMRP plays an important role in the reversible inhibition of PSD-95 mRNA translation. The loss of FMRP disables this translation control that consecutively causes an excessive production of the PSD-95 protein. The goal of this project was to investigate at the molecular level the biological role of both FMRP and microRNA in PSD-95 mRNA translation pathway. We have used different biophysical techniques to demonstrate that the PSD-95 mRNA region predicted to be bound by FMRP folds into G quadruplex structures and to analyze its binding by the protein.

Voltage-gated K Channels II

1358-Pos Board B250
Modeling HERG Isoforms
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The delayed rectifier, I_{Kr}, is formed by homo/heterotetramers of the 2 HERG splice variants HERG1a and HERG1b. The only difference between isoforms is that HERG1b has a shorter N-terminal than HERG1a. Despite their similarities, HERG1a and 1b have profoundly different gating kinetics: HERG1b has faster activation and deactivation kinetics than HERG1a. HERG activation is a complex multi-step process involving voltage-dependent and voltage independent transitions.
We used an envelope of tails protocol to isolate activation from the fast overlapping inactivation for both HERG1a and 1b. The activation rate of both HERG1a and 1b reaches an identical saturation value at very positive potentials, which suggests that the voltage-independent step is rate limiting at these potentials for both isoforms. This suggests that the voltage-insensitive step is not affected by isoform, i.e., the presence or absence of the N-terminal. At lower potentials, activation was strongly voltage dependent and was significantly different between isoforms. This indicates that the N-terminal modifies at least one voltage-sensitive step in the activation pathway. Deactivation is also strongly affected by the presence or absence of the N-terminal. This suggests that the N-terminal affects voltage-dependent transitions near the open state. Sensitivity analysis of our HERG model suggests that the effects may be more broad. Manipulation of only the early states failed to optimize the rate of deactivation, but optimization of only the final voltage dependent steps fails to recapitulate the combination of shifted steady-state activation and slope factor for the HERG1b isoform. In conclusion, our data indicate that the N-terminal interacts primarily through modification of voltage-sensitive transitions.

1359-Pos Board B251
Deletion of the Amino-Terminus Uncovers an Inactivated State in EAG1 Channels
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Ether-a-go-go (Eag) family channels are voltage-gated K^+ channels that are important in cardiac and neural function and have a well-documented role in disease. One of eight members of the mammalian Eag family, EAG1 channels expression is confined to the central nervous system of the healthy adult. EAG1 channels are also expressed in cancer cells and are implicated in tumor progression and regulation of the cell cycle. Most members of the Eag channel family exhibit voltage-dependent inactivation, yet wild-type EAG1 channels exhibit only voltage-gated activation and deactivation, with no apparent inactivation. Here we report that deletion of the entire intracellular amino-terminal domain uncovers an inactivated channel gating state at depolarizing potentials. We characterized this inactivated state in excised patches from Xenopus oocytes expressing mutant EAG1 channels, and recorded their currents in the inside-out configuration of the patch clamp technique. Similar to wild-type channels, EAG1 with a deleted amino-terminus begins to activate around −80 mV, however, these channels also inactivate at potentials higher than −40 mV. The rate of inactivation becomes faster with increasingly depolarizing potentials. Additionally, the kinetics of the EAG1 channels with a deleted amino-terminus are quite slow. Other members of the Eag family of channels exhibit C-type inactivation, which results from collapse of the selectivity filter. To determine whether the inactivation found in this EAG1 mutant was also C-type, we mutated a pore-lining residue known to alter C-type inactivation in both Shaker and hERG1 channels. As a second approach, we asked whether triethylammonium applied to the external surface of the channel altered the rate of inactivation. Results from both experiments are inconsistent with C-type inactivation. Instead, we hypothesize that inactivation in the EAG1 channel lacking their amino-terminus is simply a reclosure of the activation gate.

1360-Pos Board B252
Determining the role of the S4-S5 Linker in hERG eag Domain Regulation of Channel Gating
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Human ether-a-go-go-related gene (hERG) potassium channels exhibit unique kinetic properties, including unusually slow deactivation kinetics, which help to specialize them for their role in the heart. Previously, we demonstrated that an N-terminal eag domain is essential in regulating channel deactivation

kinetics. The mechanism by which the eag domain regulates gating remains unclear. Recent evidence suggests the intracellular loop between the S4 and S5 transmembrane domains (S4-S5 linker) may be important in regulating both activation and deactivation, and that modulation of gating by the eag domain may act via the S4-S5 linker. Here we sought to investigate the role of the S4-S5 linker using site-directed mutagenesis and a combination of electrophysiology and Förster Resonance Energy Transfer (FRET). We found that channels with alanine mutations in the S4-S5 linker exhibited altered gating. All the S4-S5 mutant channels caused an acceleration of deactivation kinetics, except for S543A which had significantly slowed deactivation. Co-expressing an eag domain gene fragment (N-eag) with S4-S5 mutant channels which additionally lacked a native eag domain (Δeag) failed to restore slow deactivation kinetics to the mutant channels. FRET analysis revealed that eag domains tagged with a CFP were in close proximity to each of the S4-S5 mutant channels tagged with a Citrine. Replacement of the entire S4-S5 linker with alanines (hERG [S4-S5]Ala) produced channels with altered gating, including fast deactivation and a far left-shifted steady-state activation curve. Co-expression of hERG Δeag[S4-S5]Ala channels with N-eag did not alter channel gating; however, FRET analysis revealed that N-eag was in close proximity to the mutant channels. Together, these findings suggest that an intact S4-S5 linker is necessary to transduce eag domain-dependent regulation of gating, but it is not required for the eag domain to bind to the channel.

1361-Pos Board B253

S4-S5 Linker Flexibility Stabilizes hERG Channel Closed States

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The hERG cardiac K^+ channel is characterized by unusually slow activation and deactivation gating kinetics compared to *Shaker*-like voltage-gated K^+ (Kv) channels. In *Shaker*-like Kv channels movement of the voltage sensor upon depolarization is mechanically transduced by the α-helical S4-S5 linker to S6 activation gate opening. Given the unique gating properties of hERG channels, the details of voltage sensor coupling with the S6 activation gate are of significant interest. We have recently shown that substitution of a glycine residue (G546) within the S4-S5 linker with a leucine residue destabilizes the closed state (left-shifts the $V_{1/2}$ of activation by ~50 mV and accelerates the rate of channel opening) and suggested that flexibility of the linker may be a key determinant of the closed-open equilibrium in hERG channels. Here, we have investigated this further by re-introducing glycine residues within the S4-S5 linker (from position 539 to 552) in the G546L background to determine whether flexibility introduced at different positions within the helix restores WT-like gating. We found a cluster of sites in the N-terminal portion of the S4-S5 linker (D540, R541, Y542, E544) that, when replaced with a glycine residue, rescued the −50 mV shift caused by the G546L mutation restoring a WT-like voltage dependence of activation. None of these mutations affected the voltage dependence of activation in the WT background. All other mutations tested did not rescue WT function and presented the destabilized closed state phenotype that is characteristic of the G546L mutation. These results suggest that flexibility of the N-terminal S4-S5 linker contributes to stabilization of hERG channels in the closed state and that the native glycine, G546, affords this flexibility in WT channels.

1362-Pos Board B254

A Proline Scan Approach to Investigate the Activation Gate of hERG Channels

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In *Shaker* channels, the activation gate is formed at the bundle crossing by the convergence of the inner S6 helices near a conserved proline-valine-proline (PVP) motif, which introduces a kink in the helices that allows for electromechanical coupling with voltage sensor motions via the S4-S5 linker. Human ether-a-go-go related gene (hERG) channels lack the PVP motif and the location of the intracellular pore gate and how it is coupled to S4 movement is less clear. Here, we performed a proline scan of the inner S6 helix, from I655 to Y667, to determine the position of the gate. The rationale was that proline-induced S6 disruption would impede gate function when a proline was engineered above, but not below, the native gate region. We discovered that proximal substitutions (I655P-Q664P) impeded gate closure trapping channels in the open state, while distal substitutions (R665P, L666P and Y667P) preserved wild type-like gating. That proline substitutions below Q664 preserved channel gating, while residues above disrupted gate function, strongly suggests that the position of the intracellular gate is formed at Q664. These data are consistent with previous homology model-based predictions[1]. Interestingly, in V659P channels the gate was trapped open, but upon strong hyperpolarization channels slowly activated into a distinct voltage-dependent open state, reminiscent of the well-studied hERG mutation, D540K. The presence of voltage-dependent gating in this mutant suggests that the trapped open phenotype is due to uncoupling of gate closure from voltage sensor gating, rather than an immobilization of sensor movement. Moreover, the activation of V659P channels upon hyperpolarization suggests a 'down' configuration of the voltage sensor that is distinct from that occupied at −80 mV and that leads to hyperpolarization-activated pore opening, as in HCN channels.

[1] Wynia-Smith et. al., J. Gen. Physiol. 132:507-520, 2008.

1363-Pos Board B255

Examining the Regulation of Voltage-Dependent S4 Movement in hERG Potassium Channels

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Unlike many Kv channels, hERG channel activation and deactivation are slow and are apparently limited by slow movement of the S4 voltage sensor. In fast-activating Shaker channels, a putative gating charge transfer centre formed in part by F290 has been proposed to interact with S4 charges (e.g. R1, K5) and, thus, modulate voltage-dependent gating. F290 is conserved in hERG (F463), but the relevant residues in the hERG S4 are reversed (K1, R5) and there is an extra positive charge adjacent to R5 (K6). We have examined whether hERG channels possess a transfer centre similar to that in Shaker and if these S4 charge differences underlie slow gating in hERG channels. Of five hERG F463 hydrophobic substitutions tested, only F463W and F463Y shifted the G-V relationship to more depolarized potentials and dramatically (>20x) slowed channel activation. With the S4 residue reversals (i.e. K1, R5) taken into account, this closed state stabilization suggests a role for F463 that is similar to that described for F290 in Shaker. Also consistent with results from Shaker, the hERG K1R mutation left-shifted the G-V relationship and stabilized the open state. In contrast to a predicted stabilization of the open state, R5K caused a moderate right-shift of the G-V and closed-state stabilization. Intriguingly, the neighbouring K6 residue was more important than R5 in hERG voltage sensing, since K6R dramatically slowed and right-shifted activation. Taken together, these data suggest a role for F463 in mediating the closed-open equilibrium, similar to that proposed for F290 in Shaker channels. Furthermore, the hERG channel open state appears to be stabilized by the unique configuration of R5 and K6 side chains. Both charges play important roles, but that of K6 appears more critical at stabilizing the activated state of the channel.

1364-Pos Board B256

Measurement of hERG Ion Channel Currents in Lipid Bilayer

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hERG channels (human ether-à -go-go related gene, Kv11.1) play an important role conducting potassium ions in the cardiac delayed rectifier current, IKr, during the repolarization phase of the cardiac action potential. We have measured hERG channels in droplet interface bilayers using membrane preparations made from eukaryotic cells expressing hERG. We find single channel conductance and reversal potentials consistent with previously published patch clamp studies as well as the sensitivity of the measured currents to astemizole, a potassium channel blocker, and E-4031, a hERG specific blocker. This sensitivity is dosage dependent, with IC50 values measured, 91 nM and 12.4 nM for astemizole and E-4031, respectively.

1365-Pos Board B257

Functional Analysis of Concatenated Heterotetrameric hERG1 Channels Reveals Requirement for Binding to Four Identical Sites to Achieve Full Activation by hERG1 Agonists

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Recently, several small molecule activators of hERG1 K^+ channels have been discovered, including PD-118057 (PD) and ICA-105574 (ICA). The putative binding sites for these two activators overlap and are located in a hydrophobic pocket between the S5 and S6 segments of adjacent hERG1 subunits. Thus, a homotetrameric hERG1 channel has potentially four identical binding sites. However, it is unclear whether drug binding to more than one site is required for full channel activation. Concatenated hERG1 tetramers containing wild-type and mutant subunits, heterologously expressed in *Xenopus* oocytes, were employed to determine the binding stoichiometry of hERG1 agonists. We previously found single mutation (L646E in S6 or F557L in S5) abolished the effects of PD and ICA, respectively. Concatenated tetramers were therefore constructed to contain a variable number (0 to 4) of the mutant subunits.